

ATTORNEY DOCKET NO. 000270-012

UNITED STATES PATENT APPLICATION

of

STEVEN L. STICE

JAMES M. ROBL

JOSE CIBELLI

and

PAUL GOLUEKE

for

CLONING PIGS USING DONOR NUCLEI FROM DIFFERENTIATED CELLS

BURNS, DOANE, SWECKER & MATHIS, L.L.P.  
P.O. BOX 1404  
ALEXANDRIA, VA 22313-1404

08888057.070397

12/13/00 *Filed 11/10/99* This application is a continuation-in-part of Serial  
No. 08/781,752, *now U.S. Patent 5,945,977 issued August 31, 1999* the contents of which are hereby  
incorporated by reference.

1. FIELD OF THE INVENTION

5 The present invention relates to cloning procedures  
in which cell nuclei derived from differentiated pig cells  
are transplanted into enucleated mammalian oocytes of the  
same species as the donor nuclei. The nuclei are  
reprogrammed to direct the development of cloned embryos,  
10 which can then be transferred into recipient females to  
produce fetuses and offspring, or used to produce cultured  
inner cell mass cells (ICM). The cloned embryos can also  
be combined with fertilized embryos to produce chimeric  
embryos, fetuses and/or offspring.

15 2. BACKGROUND OF THE INVENTION

The use of ungulate inner cell mass (ICM) cells for  
nuclear transplantation has also been reported. For  
example, Collas et al., *Mol. Reprod. Dev.*, 38:264-267  
(1994) discloses nuclear transplantation of bovine ICMs by  
20 microinjection of the lysed donor cells into enucleated  
mature oocytes. Collas et al. disclosed culturing of  
embryos *in vitro* for seven days to produce fifteen  
blastocysts which, upon transferral into bovine

recipients, resulted in four pregnancies and two births. Also, Keefer et al., *Biol. Reprod.*, 50:935-939 (1994), disclosed the use of bovine ICM cells as donor nuclei in nuclear transfer procedures, to produce blastocysts which, upon transplantation into bovine recipients, resulted in several live offspring. Further, Sims et al., *Proc. Natl. Acad. Sci., USA*, 90:6143-6147 (1993), disclosed the production of calves by transfer of nuclei from short-term *in vitro* cultured bovine ICM cells into enucleated mature oocytes.

The production of live lambs following nuclear transfer of cultured embryonic disc cells has also been reported (Campbell et al., *Nature*, 380:64-68 (1996)). Still further, the use of bovine pluripotent embryonic cells in nuclear transfer and the production of chimeric fetuses has been reported (Stice et al., *Biol. Reprod.*, 54:100-110 (1996); Collas et al, *Mol. Reprod. Dev.*, 38:264-267 (1994)). Collas et al demonstrated that granulosa cells (adult cells) could be used in a bovine cloning procedure to produce embryos. However, there was no demonstration of development past early embryonic stages (blastocyst stage). Also, granulosa cells are not easily cultured and are only obtainable from females. Collas et al did not attempt to propagate the granulosa cells in culture or try to genetically modify those cells. Wilmut et al (*Nature*, 365:810-813 (1997)) produced nuclear

transfer sheep offspring derived from fetal fibroblast cells, and one offspring from a cell derived from an adult sheep.

Cloning pig cells is more difficult in comparison with cells of other species. This phenomenon is illustrated by the following table:

0808057 070397  
262020 2508280

SPECIES (from hardest to easiest to clone)	CELL TYPE CLONED	OFFSPRING PRODUCED
Pig (Prather, 1989)	2 and 4 cell stage embryo	yes
Pig (Prather, 1989; Liu et al., 1995)	greater than 4 cell stage	no
Mouse (Cheong et al., 1993)	2,4 and 8 cell stage embryo	yes
Mouse (Tsunoda et al., 1993)	greater than 8 cell stage	no
Cattle (Keefer et al., 1994)	64 to 128 cell stage (ICM)	yes
Cattle (Stice et al., 1996)	embryonic cell line from ICM	no
Sheep (Smith et al., 1989)	64 to 128 cell stage (ICM)	yes
Sheep (Campbell et al., 1996)	embryonic cell line from ICM	yes
Sheep (Wilmut et al., 1997)	fetal and adult cells	yes

20 There also exist problems in the area of producing transgenic pigs. By current methods, heterologous DNA is introduced into either early embryos or embryonic cell lines that differentiate into various cell types in the fetus and eventually develop into a transgenic animal.

25 However, many early embryos are required to produce one transgenic animal and, thus, this procedure is very inefficient. Also, there is no simple and efficient method of selecting for a transgenic embryo before going through the time and expense of putting the embryos into

30 surrogate females. In addition, gene targeting techniques

cannot be easily accomplished with early embryo transgenic procedures.

Embryonic stem cells in mice have enabled researchers to select for transgenic cells and perform gene targeting. This allows more genetic engineering than is possible with other transgenic techniques. However, embryonic stem cell lines and other embryonic cell lines must be maintained in an undifferentiated state that requires feeder layers and/or the addition of cytokines to media. Even if these precautions are followed, these cells often undergo spontaneous differentiation and cannot be used to produce transgenic offspring by currently available methods. Also, some embryonic cell lines have to be propagated in a way that is not conducive to gene targeting procedures.

Methods for deriving embryonic stem (ES) cell lines *in vitro* from early preimplantation mouse embryos are well known. (See, e.g., Evans et al., *Nature*, 29:154-156 (1981); Martin, *Proc. Natl. Acad. Sci., USA*, 78:7634-7638 (1981)). ES cells can be passaged in an undifferentiated state, provided that a feeder layer of fibroblast cells (Evans et al., *Id.*) or a differentiation inhibiting source (Smith et al., *Dev. Biol.*, 121:1-9 (1987)) is present.

ES cells have been previously reported to possess numerous applications. For example, it has been reported that ES cells can be used as an *in vitro* model for differentiation, especially for the study of genes which

5 309:255-256 (1984)).

10 Moreover, in the case of livestock animals, e.g.,  
ungulates, nuclei from like preimplantation livestock  
embryos support the development of enucleated oocytes to  
term (Smith et al., *Biol. Reprod.*, 40:1027-1035 (1989);  
and Keefer et al., *Biol. Reprod.*, 50:935-939 (1994)).

15 This is in contrast to nuclei from mouse embryos which  
beyond the eight-cell stage after transfer reportedly do  
not support the development of enucleated oocytes (Cheong  
et al, *Biol. Reprod.*, 48:958 (1993)). Therefore, ES cells  
from livestock animals are highly desirable because they

20 may provide a potential source of totipotent donor nuclei,  
genetically manipulated or otherwise, for nuclear transfer  
procedures.

7

purportedly stable, pluripotent cell lines from pig and sheep blastocysts which exhibit some morphological and growth characteristics similar to that of cells in primary cultures of inner cell masses isolated immunosurgically from sheep blastocysts. Also, Notarianni et al., *J. Reprod. Fert. Suppl.*, 41:51-56 (1990) discloses maintenance and differentiation in culture of putative pluripotential embryonic cell lines from pig blastocysts. Gerfen et al., *Anim. Biotech*, 6(1):1-14 (1995) discloses the isolation of embryonic cell lines from porcine blastocysts. These cells are stably maintained in mouse embryonic fibroblast feeder layers without the use of conditioned medium, and reportedly differentiate into several different cell types during culture.

Further, Saito et al., *Roux's Arch. Dev. Biol.*, 201:134-141 (1992) reports cultured, bovine embryonic stem cell-like cell lines which survived three passages, but were lost after the fourth passage. Handyside et al., *Roux's Arch. Dev. Biol.*, 196:185-190 (1987) discloses culturing of immunosurgically isolated inner cell masses of sheep embryos under conditions which allow for the isolation of mouse ES cell lines derived from mouse ICMS. Handyside et al. reports that under such conditions, the sheep ICMS attach, spread, and develop areas of both ES cell-like and endoderm-like cells, but that after prolonged culture only endoderm-like cells are evident.



Also recently, Campbell et al., *Nature*, 380:64-68 (1996) reported the production of live lambs following nuclear transfer of cultured embryonic disc (ED) cells from day nine ovine embryos cultured under conditions which promote the isolation of ES cell lines in the mouse. The authors concluded that ED cells from day nine ovine embryos are totipotent by nuclear transfer and that totipotency is maintained in culture.

9

supporting the attachment and outgrowth of bovine ICM cells. They concluded that the attachment and outgrowth of cultured ICM cells is enhanced by the use of STO (mouse fibroblast) feeder cells (instead of bovine uterus  
5 epithelial cells) and by the use of charcoal-stripped serum (rather than normal serum) to supplement the culture medium. Van Stekelenburg et al reported, however, that their cell lines resembled epithelial cells more than pluripotent ICM cells.

10 Smith et al., WO 94/24274, published October 27, 1994, Evans et al, WO 90/03432, published April 5, 1990, and Wheeler et al, WO 94/26889, published November 24, 1994, report the isolation, selection and propagation of animal stem cells which purportedly may be used to obtain  
15 transgenic animals. Evans et al. also reported the derivation of purportedly pluripotent embryonic stem cells from porcine and bovine species which assertedly are useful for the production of transgenic animals. Further, Wheeler et al, WO 94/26884, published November 24, 1994,  
20 disclosed embryonic stem cells which are assertedly useful for the manufacture of chimeric and transgenic ungulates.

Thus, based on the foregoing, it is evident that many groups have attempted to produce ES cell lines, e.g., because of their potential application in the production  
25 of cloned or transgenic embryos and in nuclear transplantation.

10

## OBJECTS AND SUMMARY OF THE INVENTION

It is a more specific object of the invention to  
10 provide a novel method for cloning pigs which involves  
transplantation of the nucleus of a differentiated pig  
cell into an enucleated pig oocyte.

It is another object of the invention to provide an improved method for producing genetically engineered or transgenic pigs (i.e., NT units, fetuses, offspring). The invention also provides genetically engineered or

20 transgenic pigs, including those made by such a method.

It is a more specific object of the invention to provide a method for producing genetically engineered or transgenic pigs by which a desired DNA sequence is inserted, removed or modified in a differentiated pig cell

or cell nucleus prior to use of that differentiated cell or cell nucleus for formation of a NT unit. The invention also provides genetically engineered or transgenic pigs made by such a method

5 It is another object of the invention to provide a novel method for producing pig CICM cells which involves transplantation of a nucleus of a differentiated pig cell into an enucleated pig oocyte, and then using the resulting NT unit to produce CICM cells. The invention  
10 also provides pig CICM cells produced by such a method.

It is another object of the invention to use such pig CICM cells for therapy or diagnosis.

It is a specific object of the invention to use such pig CICM cells for treatment or diagnosis of any disease  
15 wherein cell, tissue or organ transplantation is therapeutically or diagnostically beneficial. The CICM cells may be used within the same species or across species.

It is another object of the invention to use cells or  
20 tissues derived from pig NT units, fetuses or offspring for treatment or diagnosis of any disease wherein cell, tissue or organ transplantation is therapeutically or diagnostically beneficial. Such diseases and injuries include Parkinson's, Huntington's, Alzheimer's, ALS,  
25 spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver diseases, heart disease,

It is another specific object of the invention to use cells or tissues derived from pig NT units, fetuses or offspring, or pig CICM cells produced according to the invention for the production of differentiated cells, 10 tissues or organs.

It is another object of the invention to use cells, tissues or organs produced from such tissues derived from pig NT units, fetuses or offspring, or pig CICM cells to provide improved methods of transplantation therapy. Such  
20 therapies include by way of example treatment of diseases and injuries including Parkinson's, Huntington's, Alzheimer's, ALS, spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver diseases, heart disease, cartilage replacement, burns, vascular  
25 diseases, urinary tract diseases, as well as for the

treatment of immune defects, bone marrow transplantation, cancer, among other diseases.

It is another object of the invention to provide genetically engineered or transgenic tissues derived from 5 pig NT units, fetuses or offspring, or pig CICM cells produced by inserting, removing or modifying a desired DNA sequence in a differentiated pig cell or cell nucleus prior to use of that differentiated cell or cell nucleus for formation of a NT unit.

10 It is another object of the invention to use the transgenic or genetically engineered tissues derived from pig NT units, fetuses or offspring, or pig CICM cells produced according to the invention for gene therapy, in particular for the treatment and/or prevention of the 15 diseases and injuries identified, *supra*.

It is another object of the invention to use the tissues derived from pig NT units, fetuses or offspring, or pig CICM cells produced according to the invention, or transgenic or genetically engineered tissues derived from 20 pig NT units, fetuses or offspring, or pig CICM cells produced according to the invention as nuclear donors for nuclear transplantation.

It is another object of the invention to use transgenic or genetically engineered pig offspring 25 produced according to the invention in order to produce pharmacologically important proteins.

Thus, in one aspect, the present invention provides a method for cloning a pig (e.g., embryos, fetuses, offspring). The method comprises:

(i) inserting a desired differentiated pig cell or  
5 cell nucleus into an enucleated pig oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit;

(ii) activating the resultant nuclear transfer unit;  
and

10 (iii) transferring said cultured NT unit to a host pig such that the NT unit develops into a fetus.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

15 The cells, tissues and/or organs of the fetus are advantageously used in the area of cell, tissue and/or organ transplantation, or production of desirable genotypes.

The present invention also includes a method of  
20 cloning a genetically engineered or transgenic pig, by which a desired DNA sequence is inserted, removed or modified in the differentiated pig cell or cell nucleus prior to insertion of the differentiated pig cell or cell nucleus into the enucleated oocyte. Genetically  
25 engineered or transgenic pigs produced by such a method are advantageously used in the area of cell, tissue and/or

15

organ transplantation, production of desirable genotypes, and production of pharmaceutical proteins.

Also provided by the present invention are pigs obtained according to the above method, and offspring of 5 those pigs.

In another aspect, the present invention provides a method for producing pig CICM cells. The method comprises:

- (i) inserting a desired differentiated pig cell or 10 cell nucleus into an enucleated pig oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit;
- (ii) activating the resultant nuclear transfer unit; and
- 15 (iii) culturing cells obtained from said cultured NT unit to obtain pig CICM cells.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

20 The pig CICM cells are advantageously used in the area of cell, tissue and organ transplantation.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly 25 understood by reference to the following detailed

03838057-070397  
462070 2508880



description of the preferred embodiments of the invention and to the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved procedures  
5 for cloning pigs by nuclear transfer or nuclear  
transplantation. In the subject application, nuclear  
transfer or nuclear transplantation or NT are used  
interchangeably.

According to the invention, cell nuclei derived from  
10 differentiated pig cells are transplanted into enucleated  
pig oocytes. The nuclei are reprogrammed to direct the  
development of cloned embryos, which can then be  
transferred into recipient females to produce fetuses and  
offspring, or used to produce CICM cells. The cloned  
15 embryos can also be combined with fertilized embryos to  
produce chimeric embryos, fetuses and/or offspring.

Prior art methods have used embryonic cell types in  
cloning procedures. This includes work by Campbell et al  
(*Nature*, 380:64-68, 1996) and Stice et al (*Biol. Reprod.*,  
20 54:100-110, 1996). In both of those studies, embryonic  
cell lines were derived from embryos of less than 10 days  
of gestation. In both studies, the cells were maintained  
on a feeder layer to prevent overt differentiation of the  
donor cell to be used in the cloning procedure. The  
25 present invention uses differentiated cells.

17

Adult cells and fetal fibroblast cells from a sheep have purportedly been used to produce sheep offspring (Wilmut et al, 1997). Studies have shown, however, that the cloning of pigs is more difficult than cloning sheep. In fact, of the mammalian species studied, cloning of sheep appears to be the easiest, and pig cloning appears to be the most difficult. The successful cloning of pigs using differentiated cell types according to the present invention was quite unexpected.

Thus, according to the present invention, multiplication of superior genotypes of pigs is possible. This will allow the multiplication of adult pigs with proven genetic superiority or other desirable traits. Genetic progress will be accelerated in the pig. By the present invention, there are potentially billions of fetal or adult pig cells that can be harvested and used in the cloning procedure. This will potentially result in many identical offspring in a short period.

There has also been speculation that the Wilmut et al method will lead to the generation of transgenic animals (see MacQuitty, *Nature Biotech.*, 15:294 (1997)). However, there is no reason to assume, for example, that nuclei from adult cells that have been transfected with exogenous DNA will be able to survive the process of nuclear transfer. In this regard, it is known that the properties of mouse embryonic stem (ES) cells are altered by *in vitro*

manipulation such that their ability to form viable chimeric embryos is effected. Therefore, prior to the present invention, the cloning of transgenic animals could not have been predicted.

5       The present invention also allows simplification of transgenic procedures by working with a cell source that can be clonally propagated. This eliminates the need to maintain the cells in an undifferentiated state, thus, genetic modifications, both random integration and gene  
10 targeting, are more easily accomplished. Also by combining nuclear transfer with the ability to modify and select for these cells in vitro, this procedure is more efficient than previous transgenic embryo techniques. According to the present invention, these cells can be  
15 clonally propagated without cytokines, conditioned media and/or feeder layers, further simplifying and facilitating the transgenic procedure. When transfected cells are used in cloning procedures according to the invention, transgenic pig embryos are produced which can develop into  
20 fetuses and offspring. Also, these transgenic cloned embryos can be used to produce CICM cell lines or other embryonic cell lines. Therefore, the present invention eliminates the need to derive and maintain in vitro an undifferentiated cell line that is conducive to genetic  
25 engineering techniques.

0888057 "070399" 468040" 45088880

The present invention can also be used to produce cloned pig fetuses, offspring or CICM cells which can be used, for example, in cell, tissue and organ transplantation. By taking a fetal or adult cell from a pig and using it in the cloning procedure a variety of cells, tissues and possibly organs can be obtained from cloned fetuses as they develop through organogenesis. Cells, tissues, and organs can be isolated from cloned offspring as well. This process can provide a source of "materials" for many medical and veterinary therapies including cell and gene therapy. If the cells are transferred back into the animal in which the cells were derived, then immunological rejection is averted. Also, because many cell types can be isolated from these clones, other methodologies such as hematopoietic chimerism can be used to avoid immunological rejection among animals of the same species as well as between species.

Thus, in one aspect, the present invention provides a method for cloning a pig. In general, the pig will be produced by a nuclear transfer process comprising the following steps:

- (i) obtaining desired differentiated pig cells to be used as a source of donor nuclei;
- (ii) obtaining oocytes from a pig;
- (iii) enucleating said oocytes;

(iv) transferring the desired differentiated cell or cell nucleus into the enucleated oocyte, e.g., by fusion or injection, to form NT units;

(v) activating the resultant NT unit; and

5 (vii) transferring said cultured NT unit to a host pig such that the NT unit develops into a fetus.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

10 The present invention also includes a method of cloning a genetically engineered or transgenic pig, by which a desired DNA sequence is inserted, removed or modified in the differentiated pig cell or cell nucleus prior to insertion of the differentiated pig cell or cell  
15 nucleus into the enucleated oocyte.

Also provided by the present invention are pigs obtained according to the above method, and offspring of those pigs.

In addition to the uses described above, the  
20 genetically engineered or transgenic pigs according to the invention can be used to produced a desired protein, such as a pharmacologically important protein. That desired protein can then be isolated from the milk or other fluids or tissues of the transgenic pig. Alternatively, the  
25 exogenous DNA sequence may confer an agriculturally useful trait to the transgenic pig, such as disease resistance,

decreased body fat, increased lean meat product, improved feed conversion, or altered sex ratios in progeny.

The present invention further provides for the use of NT fetuses and NT and chimeric offspring in the area of cell, tissue and organ transplantation.

In another aspect, the present invention provides a method for producing pig CICM cells. The method comprises:

- (i) inserting a desired differentiated pig cell or cell nucleus into an enucleated pig oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit;
- (ii) activating the resultant nuclear transfer unit; and
- (iii) culturing cells obtained from said cultured NT unit to obtain pig CICM cells.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

The pig CICM cells are advantageously used in the area of cell, tissue and organ transplantation, or in the production of fetuses or offspring, including transgenic fetuses or offspring.

As used herein, a fetus is the unborn young of a viviporous animal after it has taken form in the uterus. In pigs, the fetal stage occurs from 30 days after

conception until birth. A mammal is an adult from birth until death.

Preferably, the NT units will be cultured to a size of at least 2 to 400 cells, preferably 4 to 128 cells, and most preferably to a size of at least about 50 cells.

Nuclear transfer techniques or nuclear transplantation techniques are known in the literature and are described in many of the references cited in the Background of the Invention. See, in particular, Campbell et al, *Theriogenology*, 43:181 (1995); Collas et al, *Mol. Report Dev.*, 38:264-267 (1994); Keefer et al, *Biol. Reprod.*, 50:935-939 (1994); Sims et al, *Proc. Natl. Acad. Sci., USA*, 90:6143-6147 (1993); WO 94/26884; WO 94/24274, and WO 90/03432, which are incorporated by reference in their entirety herein. Also, U.S. Patent Nos. 4,944,384 and 5,057,420 describe procedures for bovine nuclear transplantation.

Differentiated refers to cells having a different character or function from the surrounding structures or from the cell of origin. Differentiated pig cells are those cells which are past the early embryonic stage. More particularly, the differentiated cells are those from at least past the embryonic disc stage (day 10 of bovine embryogenesis). The differentiated cells may be derived from ectoderm, mesoderm or endoderm.

Pig cells may be obtained by well known methods. Pig cells useful in the present invention include, by way of example, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. Moreover, the pig cells used for nuclear transfer may be obtained from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc. These are just examples of suitable donor cells. Suitable donor cells, i.e., cells useful in the subject invention, may be obtained from any cell or organ of the body. This includes all somatic or germ cells.

Fibroblast cells are an ideal cell type because they can be obtained from developing fetuses and adult pigs in large quantities. Fibroblast cells are differentiated somewhat and, thus, were previously considered a poor cell type to use in cloning procedures. Importantly, these cells can be easily propagated in vitro with a rapid doubling time and can be clonally propagated for use in gene targeting procedures. Again the present invention is novel because differentiated cell types are used. The present invention is advantageous because the cells can be



easily propagated, genetically modified and selected in vitro.

Methods for isolation of oocytes are well known in the art. Essentially, this will comprise isolating 5 oocytes from the ovaries or reproductive tract of a pig. A readily available source of pig oocytes is slaughterhouse materials.

For the successful use of techniques such as genetic engineering, nuclear transfer and cloning, oocytes must 10 generally be matured *in vitro* before these cells may be used as recipient cells for nuclear transfer, and before they can be fertilized by the sperm cell to develop into an embryo. This process generally requires collecting immature (prophase I) oocytes from pig ovaries, e.g., pig 15 ovaries obtained at a slaughterhouse, and maturing the oocytes in a maturation medium prior to fertilization or enucleation until the oocyte attains the metaphase II stage, which in the case of pig oocytes generally occurs about 35-45 hours post-aspiration. For purposes of the 20 present invention, this period of time is known as the "maturation period." As used herein for calculation of time periods, "aspiration" refers to aspiration of the immature oocyte from ovarian follicles.

Additionally, metaphase II stage oocytes, which have 25 been matured *in vivo* have been successfully used in nuclear transfer techniques. For example, mature

metaphase II oocytes have been collected surgically from either non-superovulated or superovulated cows or heifers 35 to 48 hours past the onset of estrus or past the injection of human chorionic gonadotropin (hCG) or 5 similar hormone. Similar procedures can be used in pigs.

The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be significant to the success of NT methods. (See e.g., Prather et al., *Differentiation*, 48, 1-8, 1991). In general, successful 10 mammalian embryo cloning practices use the metaphase II stage oocyte as the recipient oocyte because at this stage it is believed that the oocyte can be or is sufficiently "activated" to treat the introduced nucleus as it does a fertilizing sperm. In domestic animals, the oocyte 15 activation period generally ranges from about 16-52 hours, preferably about 35-45 hours post-aspiration.

For example, immature oocytes may be washed in maturation medium (MAT -- see Table in the Examples). The oocytes are then placed in 1 to 2 mls of MAT and cultured 20 for 22 hours in the presence of db-cAMP and hormones. The oocytes are washed again, followed by culturing in MAT without hormones for an additional 18 hours.

After a maturation period, which ranges from about 30 to 50 hours, and preferably about 40 hours, the oocytes 25 will be enucleated. Prior to enucleation the oocytes will preferably be removed and placed in HECM (Seshagiri and

Bavister, 1989) containing 1 milligram per milliliter of hyaluronidase prior to removal of cumulus cells. This may be effected by repeated pipetting through very fine bore pipettes or by vortexing briefly (about 3 minutes). The 5 stripped oocytes are then screened for polar bodies, and the selected metaphase II oocytes, as determined by the presence of polar bodies, are then used for nuclear transfer. Enucleation follows.

Enucleation may be effected by known methods, such as 10 described in U.S. Patent No. 4,994,384 which is incorporated by reference herein. For example, metaphase II oocytes are either placed in HECM, optionally containing 7.5 micrograms per milliliter cytochalasin B (CB) and 0.15 M sucrose, for immediate enucleation, or may 15 be placed in a suitable medium, for example an embryo culture medium such as NCSU 23 (see Table in the Examples) at 39°C and 5% CO<sub>2</sub>, and then enucleated later, preferably not more than 24 hours later, and more preferably immediately.

20 Enucleation may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm. The oocytes are screened to identify those of which have been successfully enucleated. This screening may be effected by staining the oocytes with 1 microgram 25 per milliliter 33342 Hoechst dye for 20 min in NCSU 23, and then viewing the oocytes under ultraviolet irradiation

for less than 10 seconds. The oocytes that have been successfully enucleated can then be placed in a suitable culture medium, e.g., HECM and 0.15 M sucrose.

In the present invention, the recipient oocytes will preferably be enucleated at a time ranging from about 30 hours to about 50 hours after the initiation of *in vitro* maturation, more preferably from about 38 hours to about 42 hours after initiation of *in vitro* maturation, and most preferably about 40 hours after initiation of *in vitro* maturation.

A single pig cell will then be transferred into the perivitelline space of the enucleated oocyte used to produce the NT unit. The pig cell and the enucleated oocyte will be used to produce NT units according to methods known in the art. For example, the cells may be fused by electrofusion. Electrofusion is accomplished by providing a pulse of electricity that is sufficient to cause a transient breakdown of the plasma membrane. This breakdown of the plasma membrane is very short because the membrane reforms rapidly. Thus, if two adjacent membranes are induced to breakdown and upon reformation the lipid bilayers intermingle, small channels will open between the two cells. Due to the thermodynamic instability of such a small opening, it enlarges until the two cells become one. Reference is made to U.S. Patent 4,997,384 by Prather et al., (incorporated by reference in its entirety herein)

for a further discussion of this process. A variety of electrofusion media can be used including e.g., sucrose, mannitol, sorbitol and phosphate buffered solution.

Fusion can also be accomplished using Sendai virus as a fusogenic agent (Graham, *Wister Inot. Symp. Monogr.*, 9, 19, 1969). A preferred fusion medium is 0.28 M mannitol, 10  $\mu$ M  $\text{CaCl}_2$ , 100  $\mu$ M  $\text{MgSO}_4$  and 10 mM histidine, pH 7.0.

Also, in some cases (e.g. with small donor nuclei) it may be preferable to inject the nucleus directly into the oocyte rather than using electroporation fusion. Such techniques are disclosed in Collas and Barnes, *Mol. Reprod. Dev.*, 38:264-267 (1994), incorporated by reference in its entirety herein.

Prior to introduction into the fusion chamber, the NT units are preferably gradually exposed to the fusion medium via 3 incubations containing HECM to fusion medium in ratios of 2:1, 1:2 and 0:1. Preferably, the pig cell and oocyte are electrofused in a 500  $\mu$ m chamber by application of an electrical pulse of 90-120V for about 30  $\mu$ sec, about 44 hours after initiation of oocyte maturation. After fusion, the resultant fused NT units are maintained in fusion medium for 5 min, then placed in HECM for 10 min, and then in NCSU 23 plus 7.5 mg/ml CB until activation. Typically activation will be effected shortly thereafter, typically less than 24 hours later, and preferably about 4-9 hours later.

The NT unit may be activated by known methods. Such methods include, e.g., culturing the NT unit at sub-physiological temperature, in essence by applying a cold, or actually cool temperature shock to the NT unit. This may be most conveniently done by culturing the NT unit at room temperature, which is cold relative to the physiological temperature conditions to which embryos are normally exposed.

In a preferred embodiment, the pig NT units are activated in a 500  $\mu$ m chamber by application of an electrical pulse of 30V for 30  $\mu$ sec in an activation medium containing 0.28 M mannitol, 100  $\mu$ M  $\text{CaCl}_2$ , 100  $\mu$ M  $\text{MgSO}_4$  and 10 mM histidine, pH 7.0. One hour later a second pulse of 15V is applied for 30  $\mu$ sec. Between pulses the NT units are maintained in NCSU 23 with CB at 39°C and 5%  $\text{CO}_2$ .

Alternatively, activation may be achieved by application of known activation agents. For example, penetration of oocytes by sperm during fertilization or the activation factor contained in sperm cells can activated NT units. Also, treatments such as electrical or chemical shock, calcium ionophores, and protein kinase inhibitors may be used to activate NT embryos after fusion.

Preferably, after activation the NT units are cultured for 3 to 4 hours in NCSU 23 plus CB, and

thereafter in NCSU 23 without CB. The NT units can be transferred into the recipient female anytime after activation.

Alternatively, the activated NT units may then be  
5 cultured in a suitable *in vitro* culture medium until the generation of CICM cells and cell colonies. Culture media suitable for culturing and maturation of embryos are well known in the art. Examples of known media include Ham's F-10 + 10% fetal calf serum (FCS), Tissue Culture Medium-  
10 199 (TCM-199) + 10% fetal calf serum, Tyrodes-Albumin-Lactate-Pyruvate (TALP), Dulbecco's Phosphate Buffered Saline (PBS), Eagle's and Whitten's media. One of the most common media used for the collection and maturation of oocytes is TCM-199, and 1 to 20% serum supplement  
15 including fetal calf serum, newborn serum, estrual cow serum, lamb, pig, or steer serum. A preferred maintenance medium includes TCM-199 with Earl salts, 10% fetal calf serum, 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulphate. More preferably, the medium used is NCSU 23,  
20 and 2 to 5 days after activation the NT units are cultured in fresh NCSU 23 and 5 to 10% fetal calf serum. Any of the above may also involve co-culture with a variety of cell types such as granulosa cells, oviduct cells, BRL cells and uterine cells and STO cells.

25 Another maintenance medium is described in U.S. Patent 5,096,822 to Rosenkrans, Jr. et al., which is

incorporated herein by reference. This embryo medium, named CR1, contains the nutritional substances necessary to support an embryo.

Typically, the NT units are cultured in NCSU 23 plus 5 to 10% FCS until the NT units reach a size suitable for transferring to a recipient female, or for obtaining cells which may be used to produce CICM cells or cell colonies. Preferably, these NT units will be cultured until at least about 2 to 400 cells, more preferably about 4 to 128 cells, and most preferably at least about 50 cells. The culturing will be effected under suitable conditions, i.e., about 38.5°C and 5% CO<sub>2</sub>, with the culture medium changed in order to optimize growth typically about every 2-5 days, preferably about every 3 days.

The methods for embryo transfer and recipient animal management in the present invention are standard procedures used in the embryo transfer industry. Synchronous transfers are important for success of the present invention, i.e., the stage of the NT embryo is in synchrony with the estrus cycle of the recipient female. This advantage and how to maintain recipients are discussed in Wall et al ("Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei," *Biol. Reprod.*, 32:645-651 (1985)), the contents of which are hereby incorporated by reference.



Any known method for inserting, deleting or modifying a desired DNA sequence from a mammalian cell may be used for altering the differentiated cell to be used as the nuclear donor. These procedures may remove all or part of a DNA sequence, and the DNA sequence may be heterologous. Included is the technique of homologous recombination, which allows the insertion, deletion or modification of a DNA sequence or sequences at a specific site or sites in the cell genome.

33

having improved meat production, reproductive traits and disease resistance. Furthermore, cell and tissues from the NT fetus, including transgenic and/or chimeric fetuses, can be used in cell, tissue and organ

5 transplantation for the treatment of numerous diseases as described below in connection with the use of CICM cells. Hence, transgenic pigs have uses including models for diseases, xenotransplantation of cells and organs, and production of pharmaceutical proteins.

10 For production of CICM cells and cell lines, after NT units of the desired size are obtained, the cells are mechanically removed from the zone and are then used. This is preferably effected by taking the clump of cells which comprise the NT unit, which typically will contain  
15 at least about 50 cells, washing such cells, and plating the cells onto a feeder layer, e.g., irradiated fibroblast cells. Typically, the cells used to obtain the stem cells or cell colonies will be obtained from the inner most portion of the cultured NT unit which is preferably at  
20 least 50 cells in size. However, NT units of smaller or greater cell numbers as well as cells from other portions of the NT unit may also be used to obtain ES cells and cell colonies. The cells are maintained in the feeder layer in a suitable growth medium, e.g., alpha MEM  
25 supplemented with 10% FCS and 0.1 mM  $\beta$ -mercaptoethanol (Sigma) and L-glutamine. The growth medium is changed as

often as necessary to optimize growth, e.g., about every 2-3 days.

5 This culturing process results in the formation of C1CM cells or cell lines. One skilled in the art can vary the culturing conditions as desired to optimize growth of the particular C1CM cells. Also, genetically engineered or transgenic pig C1CM cells may be produced according to the present invention. That is, the methods described above can be used to produce NT units in which a desired DNA sequence or sequences have been introduced, or from which all or part of an endogenous DNA sequence or sequences have been removed or modified. Those genetically engineered or transgenic NT units can then be used to produce genetically engineered or transgenic C1CM 15 cells.

The resultant C1CM cells and cell lines have numerous therapeutic and diagnostic applications. Most especially, such C1CM cells may be used for cell transplantation therapies.

20 In this regard, it is known that mouse embryonic stem (ES) cells are capable of differentiating into almost any cell type, e.g., hematopoietic stem cells. Therefore, pig C1CM cells produced according to the invention should possess similar differentiation capacity. The C1CM cells 25 according to the invention will be induced to differentiate to obtain the desired cell types according

to known methods. For example, the subject pig CICM cells may be induced to differentiate into hematopoietic stem cells, neural cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, neural cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of CICM cells are known in the art as are suitable culturing conditions.

10 For example, Palacios et al, *Proc. Natl. Acad. Sci., USA*, 92:7530-7537 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a  
15 suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, *J. Reprod. Fertil. Dev.*, 6:543-  
20 552 (1994) is a review article which references numerous articles disclosing methods for *in vitro* differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

25 Further, Bain et al, *Dev. Biol.*, 168:342-357 (1995) teaches *in vitro* differentiation of embryonic stem cells

to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem cells. These references and in particular the disclosures  
5 therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject CICM cells,  
10 including genetically engineered or transgenic CICM cells, to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc.

The subject CICM cells may be used to obtain any desired differentiated cell type. Therapeutic usages of  
15 such differentiated cells are unparalleled. For example, hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases  
20 that compromise the immune system, such as AIDS.

Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, obtaining CICM cells as described above, and culturing  
25 such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such

hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The present invention can be used to replace defective genes, e.g., defective immune system genes, or  
5 to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc.

DNA sequences which may be introduced into the subject CICM cells include, by way of example, those which  
10 encode epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin-3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokines (interleukins, interferons, colony stimulating  
15 factors, tumor necrosis factors (alpha and beta), etc.), therapeutic enzymes, etc.

The present invention includes the use of pig cells in the treatment of human diseases. Thus, pig CICM cells, NT fetuses and NT and chimeric offspring (transgenic or  
20 non-transgenic) may be used in the treatment of human disease conditions where cell, tissue or organ transplantation is warranted. In general, CICM cell, fetuses and offspring according to the present invention can be used within the same species (autologous, syngenic  
25 or allografts) or across species (xenografts). For

Also, the subject C1CM cells, may be used as an *in vitro* model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Also, differentiated cell tissues and organs using the subject C1CM cells may be used in drug studies.

Further, the subject C1CM cells may be used as  
10 nuclear donors for the production of other C1CM cells and  
cell colonies.

In order to more clearly describe the subject invention, the following examples are provided.

EXAMPLES

**MATERIALS AND METHODS FOR PIG CLONING**

**Modified NCSU 37 Medium (mNCSU 37)**

Component	Mol. Wt.	Conc. (mM)	g/l
NaCl	58.45	108.73	6.3553
NaHCO <sub>3</sub>	84.00	25.07	2.1059
KCl	74.55	1.78	0.3563
KH <sub>2</sub> PO <sub>4</sub>	136.09	1.19	0.1619
MgSO <sub>4</sub> ·7H <sub>2</sub> O	246.50	1.19	0.2933
CaCl <sub>2</sub> ·2H <sub>2</sub> O	147.00	1.70	0.2499
Glucose	180.20	5.55	1.0000
Glutamine	146.10	1.70	0.1461
Sorbitol	182.20	12.00	2.1864
Insulin	----	5 mg/l	0.0050
Penicillin G	----	100 IU/l	0.0650
Streptomycin	----	50 mg/l	0.0500

Use 18 mohm, RO, DI water.

pH should be 7.4, Check osmolarity and record.

Sterilize by vacuum filtration (0.22 μm), date and initial bottle.

Store at 4°C and use within 10 days.



**Modified TL-Hepes-PVA Medium (Hepes-PVA)**

Component	Mol. Wt.	Conc. (mM)	g/l
NaCl	58.45	114.00	6.6633
KCl	74.55	3.20	0.2386
5 NaHCO <sub>3</sub>	84.00	2.00	0.1680
NaH <sub>2</sub> PO <sub>4</sub>	120.00	0.34	0.0408
Na Lactate**	112.10	10.00	1.868 ml
MgCl <sub>2</sub> ·6H <sub>2</sub> O	203.30	0.50	0.1017
CaCl <sub>2</sub> ·2H <sub>2</sub> O*	147.00	2.00	0.2940
10 Sorbitol	182.20	12.00	2.1864
HEPES	238.30	10.00	2.3830
Na Pyruvate	110.00	0.20	0.0220
Gentamycin	----	----	500 µl
Penicillin G	----	----	0.0650
15 PVA	10,000	----	0.1000

\*\*60% syrup

\* Add CaCl<sub>2</sub>·2H<sub>2</sub>O last, slowly to prevent precipitation

Use 18 mohm, RO, DI water.

Adjust pH to 7.4, Check osmolarity and record.

20 Sterilize by vacuum filtration (0.22 µm), date and initial bottle.

Store at 4°C and use within 10 days.

70410

26E020" 5088880

41

**NCSU 23 Medium**

Component	Mol. Wt.	Conc. (mM)	g/l
NaCl	58.45	108.73	6.3553
NaHCO <sub>3</sub>	84.00	25.07	2.1059
KCl	74.55	4.78	0.3563
KH <sub>2</sub> PO <sub>4</sub>	136.09	1.19	0.1619
MgSO <sub>4</sub> ·7H <sub>2</sub> O	246.50	1.19	0.2933
CaCl <sub>2</sub> ·2H <sub>2</sub> O	147.00	1.70	0.2499
Glucose	180.20	5.55	1.0000
Glutamine	146.10	1.00	0.1461
Taurine	125.10	7.00	0.8757
Hypotaurine	109.10	5.00	0.5455
BSA	----	0.4%	4.0000
Penicillin G	----	100 IU/1	0.0650
Streptomycin	----	50 mg/l	0.0500

Use 18 mohm, RO, DI water.

pH should be 7.4, Check osmolarity and record.

Sterilize by vacuum filtration (0.22 µm) using red Nalgene filters, date and initial bottle.

20 Store at 4°C and use within 10 days.

NOTE: BSA type is important. Preferably use Sigma BSA catalog # A-7906. Also, Pen G/Strept is optional.

42

### Media Preparation

#### Maturation Medium (MAT):

18.0 ml mNCSU 37

2.0 ml porcine follicular fluid (pFF)

5 7.0  $\mu$ l of diluted  $\beta$ -Mercaptoethanol (dilute 10  $\mu$ l  $\beta$ -Mercaptoethanol to 990  $\mu$ l mNCSU 37; 50  $\mu$ M final concentration)

0.002 g cysteine (0.6 mM final concentration)

20  $\mu$ l EGF Stock (Epidermal Growth Factor from 10 ng/ $\mu$ l EGF  
10 stock)

Filter through 0.22  $\mu$ m into 10 ml culture tubes. Label with date and initials, equilibrate in CO<sub>2</sub> incubator.

#### Porcine Follicular Fluid Preparation

Collect follicular fluid from 3-6 mm follicles of  
15 prepubertal gilts and allow oocytes and follicular cells to settle for 5-10 minutes. Aspirate the pFF and move to 15 ml conical tubes. Centrifuge on Sorvall at 4°C at 3000 rpm for 30 minutes. Remove tubes, collect pFF above pellet, pool and filter through a 0.8  $\mu$ m, then a 0.45  $\mu$ m  
20 filter (Sterivex). Aliquot to 1.5 ml sterile microfuge tubes and freeze at -20°C until use.

**Epidermal Growth Factor Stock (EGF)**

100  $\mu$ g EGF

10 ml mNCSU 37 with 0.1% BSA

Mix well. Aliquot to 25  $\mu$ l, freeze at -20°C.

**5 Equine Chorionic Gonadotropin and human Chorionic  
Gonadotropin Stock for MAT (PMSG/hCG)**

ECG (PMSG 6000; Intervet Inc., Millsboro; DE 19966)

Dilute 6000 IU to 2000 IU/ml by adding 3 ml dH<sub>2</sub>O.

hCG (Chorulon; Intervet Inc.)

10 Dilute 10,000 IU to 2000 IU/ml by adding 5 ml dH<sub>2</sub>O.

Mix 1 ml PMSG and 1 ml hCG to get 1000 IU/ml of each

hormone. Make 50  $\mu$ l aliquots and freeze at -20°C. Freeze  
remaining PMSG and hCG stocks as well.

**db-cAMP 100 mM Stock**

15 25 mg db-cAMP (stored in dessicator at -20°C)

0.509 ml dH<sub>2</sub>O

Mix well. Make 50  $\mu$ l aliquots and freeze at -20°C.

**Fusion Medium**

0.28 M Mannitol

20 10  $\mu$ M CaCl<sub>2</sub>

100  $\mu$ M MgSO<sub>4</sub>

10 mM Histidine

adjust to pH 7.0

**Activation Medium**

0.28 M Mannitol  
100  $\mu$ M  $\text{CaCl}_2$   
100  $\mu$ M  $\text{MgSO}_4$   
5 10 mM Histidine  
adjust to pH 7.0

**Antibiotic/Antimycotic (Ab/Am)**

100 U/l Penicillin, 100  $\mu$ g/l streptomycin and 0.25  $\mu$ g/l  
amphotericin B, (Gibco #15240-062)  
10 Add a 10 ml aliquot to each liter of saline.  
Add 10  $\mu$ l to each ml of semen.

**Oocyte-Cumulus Complex (OCC) Collection**

Ovaries are transported to the lab at 25°C and  
immediately washed with 0.9% saline with  
15 antibiotic/antimycotic (10 ml/L; Gibco #600-5240g).  
Follicles between 3-6 mm are aspirated using 18g needles  
and 50 ml Falcon tubes connected to vacuum system (GEML  
bovine system). After tube is filled, OCC's are allowed  
to settle for 5-10 minutes. Follicular fluid (pFF) is  
20 aspirated and saved for use in culture system if needed  
(see pFF preparation protocol below).

0803057-070397

u5

### **OCC Washing**

OCCs are resuspended in 20 ml Hepes-PVA and allowed to settle; repeat 2 times. After last wash, OCCs are moved to grid dishes and selected for culture. Selected 5 OCCs are washed twice in 60 mm dishes of Hepes-PVA. All aspiration and oocyte recovery are performed at room temperature (approx. 25°C).

### **In Vitro Maturation (IVM)**

After washing 3 times in MAT, 50 OCCs are moved to 10 0.5 ml of MAT in 4 well Nunc plate (inner compartment contains 1-2 ml MAT or mNCSI 37). Add 5  $\mu$ l of 100 mM db-cAMP (in water) to each well of OCC. Culture 22 hours with hormones. Wash 3 times with fresh MAT without hormones and move to 0.5 ml wells of fresh MAT, 15 approximately 50 oocytes/well. Incubate for 22 hours at 39°C in 5.0% CO<sub>2</sub> atmosphere for a total of approximately 40 hours in MAT.

### **Isolation of primary cultures of porcine embryonic and adult fibroblast cells**

20 Primary cultures of porcine fibroblasts are obtained from pig fetuses 30 to 114 days postfertilization, preferably 35 days. The head, liver, heart and alimentary tract are aseptically removed, the fetuses minced and incubated for 30 minutes at 37°C in prewarmed trypsin EDTA

solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells are plated in tissue culture dishes and cultured in fibroblast growth medium (FGM) containing: alpha-MEM medium (BioWhittaker, Walkersville, MD)

5 supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50  $\mu$ l/ml). The fibroblasts are grown and maintained in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C.

Adult fibroblast cells are isolated from the lung and  
10 skin of a pig. Minced lung tissue is incubated overnight at 10°C in trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). The following day tissue and any disassociated cells are incubated for one hour at 37°C in prewarmed trypsin EDTA solution (0.05%  
15 trypsin/0.02% EDTA; GIBCO, Grand Island, NY) and processed through three consecutive washes and trypsin incubations (one hr). Fibroblast cells are plated in tissue culture dishes and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum  
20 (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50  $\mu$ l/ml). The fibroblast cells can be isolated at virtually any time in development, ranging from approximately post embryonic disc stage through adult life of the animal (porcine day 9 to 10 after  
25 fertilization to 5 years of age or longer).

U<sup>x</sup>

### **Preparation of fibroblast cells for nuclear transfer**

Examples of fetal fibroblasts which may be used as donor nuclei are:

1. Proliferating fibroblast cells that are not  
5 synchronized in any one cell stage or serum starved or  
quiescent can serve as nuclear donors. The cells from the  
above culture are treated for 10 minutes with trypsin EDTA  
and are washed three times in 100% fetal calf serum.  
Single cell fibroblast cells are then placed in  
10 micromanipulation drops of HbT medium (Bavister et al.,  
1983). This is done 10 to 30 min prior to transfer of the  
fibroblast cells into the enucleated pig oocyte.  
Preferably, proliferating transgenic fibroblast cells  
having the CMV promoter and green fluorescent protein gene  
15 (9th passage) are used to produce NT units.

2. By a second method, fibroblast cells are  
synchronized in G1 or G0 of the cell cycle. The  
fibroblast cells are grown to confluence. Then the  
concentration of fetal calf serum in the FGM is cut in  
20 half over four consecutive days (day 0 = 10%, day 1 = 5%,  
day 2 = 2.5%, day 3 = 1.25%, day 4 = 0.625%. On the fifth  
day the cells are treated for 10 minutes with trypsin EDTA  
and washed three times in 100% fetal calf serum. Single  
cell fibroblasts are then placed in micromanipulation  
25 drops of HbT medium. This is done within 15 min prior to



transfer of the fibroblast cells into the enucleated pig oocyte.

#### **Removal of cumulus cells**

After a maturation period, which ranges from about 30  
5 to 50 hours, and preferably about 40 hours, the oocytes  
will be enucleated. Prior to enucleation the oocytes will  
preferably be removed and placed in HECM (Seshagiri and  
Bavister, 1989) containing 1 milligram per milliliter of  
hyaluronidase prior to removal of cumulus cells. This may  
10 be effected by repeated pipetting through very fine bore  
pipettes or by vortexing briefly (about 3 minutes). The  
stripped oocytes are then screened for polar bodies, and  
the selected metaphase II oocytes, as determined by the  
presence of polar bodies, are then used for nuclear  
15 transfer. Enucleation follows.

#### **Enucleation, transfer of fibroblast cells and fusion**

The cumulus free pig oocytes are enucleated with a  
beveled micropipette at approximately 40 hrs post  
maturation (hpm). This procedure has been described  
20 previously by Prather et al., 1989, the contents of which  
are hereby incorporated by reference. The oocytes are  
enucleated in HECM HEPES and 7.5 mg/ml CB plus 0.15 M  
sucrose. Enucleation is confirmed after the oocytes have  
been incubated for greater than 20 minutes in NCSU 23

medium plus Hoechst 3342 (3  $\mu$ g/ml; Sigma). Individual donor cells (fibroblasts) are then placed in the perivitelline space of the recipient oocyte using the beveled micropipette in HECM HEPES plus 0.15 M sucrose and 5 CB (7.5 mg/ml). The porcine oocyte cytoplasm and the donor nucleus (NT unit) are fused together using electrofusion techniques. The NT units are washed three times in increasing amounts of fusion medium (ratio of HECM HEPES to fusion medium of 2:1, 1:2 and 0:1). The 10 fusion chamber consists of two wires of 200  $\mu$ m in diameter running in parallel with a gap of 500  $\mu$ m. Each NT unit is manually aligned so that the membranes to be fused are parallel to the two wires. One fusion pulse consisting of 100 V for 30  $\mu$ sec is applied to the NT units in an 15 electrofusion chamber. This occurs at 44 to 45 hpm. NT units are incubated in fusion medium for 5 min and then in HECM HEPES for 10 min. The NT units are placed back into NCSU 23 plus CB medium until 47 to 49 hpm.

#### Activation

20 Examples of methods of activation which may be used at 47 to 49 hrs post activation are:

1. *Single activation pulse.* NT units are removed from the NCSU 23 plus CB and washed three times in activation medium. After equilibration, the NT units are 25 placed into the fusion chamber (500  $\mu$ m gap) filled with

activation medium as described in the fusion procedure. A pulse of 30 V for 30  $\mu$ sec is applied. Then the NT units are immediately washed three times in HECM HEPES and cultured (39°C, 5% CO<sub>2</sub>) in NCSU 23 for 2 more hours until embryo transfer or in vitro culture (39°C, 5% CO<sub>2</sub> in NCSU 23). If cultured, NT units are placed in fresh NCSU 23 plus 5% fetal calf serum on day 2 of culture. The results in Table 1 indicate that oocytes can be activated using this procedure and that they have developmental capabilities.

2. *Two activation pulses.* NT units are removed from the NCSU 23 plus CB and washed three times in activation medium. After equilibration the NT units are placed back into the fusion chamber (500  $\mu$ m gap) filled with activation medium as described in the fusion procedure. A pulse of 30 V for 30  $\mu$ sec is applied. Then the NT units are immediately washed three times in HECM HEPES, placed back in NCSU 23 plus CB, and cultured in this at 39°C, 5% CO<sub>2</sub>, until the next electrical pulse 1 hr later. After 1 hr this time the activation medium equilibration step is repeated and a pulse of 15 V for 30  $\mu$ sec is applied. Then the NT units are immediately washed three times in HECM HEPES, placed back in NCSU 23 plus CB, and cultured in this medium at 39°C, 5% CO<sub>2</sub>, for 2 to 6 more hours. The NT units are then cultured using the same procedure described above in 1. The results in Table 1 indicate that oocytes

can be activated using this procedure and that they have developmental capabilities. The same is true for nuclear transfer embryos. Four blastocyst stage NT units were produced with the two pulse activation procedure.

5        3. *Sperm factor*. First described in mammalian sperm by Stice and Robl (*Mol. Reprod. Dev.*, 25:272-280 (1990)) (the contents of which are hereby incorporated by reference), this factor causes activation in oocytes. The method of sperm factor isolation from pig sperm cells and  
10 microinjection is described in Fissore et al. (*Mol. Reprod. Dev.*, 46:176-189 (1997)), the contents of which are hereby incorporated by reference. NT units are removed from the NCSU 23 plus CB and placed in micromanipulation plates described above for enucleation  
15 and fibroblast transfer. Using a micro-injection needle (1  $\mu$ m opening) filled with sperm factor the oocytes undergo activation after the delivery of the factor into the cytoplasm of the NT unit. After microinjection, the NT embryos are washed in HECM HEPES and held in NCSU 23 plus CB for 2 to 6 hours, and thereafter in NCSU 23 until embryo transfer.

52

Table 1. Development of activated oocytes and NT units using different activation procedures.

	number given activation stimulus	number cleaved (began to develop) [%]	number to blastocyst stage (eight day old embryos) [%]
Single pulse oocytes	52	6 [12]	1 [2]
Double pulse oocytes	85	8 [10]	3 [4]
Double pulse NT units	55	10 [18]	4 [7]
Sperm-factor oocytes	49	4 [8]	2 [4]

#### Embryo transfer

Methods of embryo one cell embryo transfer in pigs are well known (see, for example, Pinkert et al., 1993, the contents of which are hereby incorporated by reference). Briefly, 20 to 30 NT units are synchronously transferred into the oviduct of bred or unbred gilts. After and beyond 29 days of gestation, nuclear transfer fetuses (transgenic or non-transgenic) can be recovered from the recipient gilt. Alternatively, the fetuses are allowed to go to term (114 day gestation) and cloned pig offspring are produced.